C1. PROJECT TITLE: Answering longstanding plant ecology questions with new technology: the effects of changes in leaf proteins with age

C2. AIMS AND BACKGROUND

Total leaf nitrogen is a quantity widely used for many purposes in ecology. N concentration in mature leaves is a major predictor for photosynthesis (*1, 2*) and respiration (*3, 4*), because photosynthesis proteins make up a large fraction of leaf N and because protein turnover is a major source of respiration costs. Leading forest models revolve around the interplay between C and N currencies (*5-7*). C:N:P stoichiometry has been invoked as an expression of growth strategy (*12*), since RNA is P-rich compared to photosynthetic proteins that are N-rich. N:P stoichiometry in leaves and leaf litter varies strongly with latitude as well as between sites and species, increasing steeply towards the tropics (*9, 13, 14*). Litter decomposition rates are strongly influenced by species leaf traits, including N (*15*). Low leaf N is associated with lower food quality for herbivores, and plant N distribution influences herbivore behaviour and specialization (*16, 17*). Leaf N is often marginal for folivore growth (*18-21*) and the resistance of leaf proteins to enzymatic degradation reduces leaf nutritional value (*22-24*). Because elevated CO_2 will increase the carbon-capture efficiency of photosynthetic proteins in C_3 plants, climate change is expected to increase C:N ratios in plant tissues, with cascading consequences for all these ecosystem interactions as well as for primary production (*25*). This project is about improving our understanding leaf N through proteomics, which has large implications for ecology on all scales.

Total leaf N implicitly treats tens of thousands of proteins and other forms of N as a single pool. Researchers have been well aware total N is a mixture of different pools with different functions (e.g. (*26, 27*), but pools have only occasionally been distinguished for ecological purposes (e.g. (*28-32*)). The present proposal innovates in two main ways:

- **Recently developed proteomics methods together with gene ontologies are applied to quantify changing N distribution among protein categories, particularly stress- and defense- versus photosynthesis-related proteins.**
- **Proteomics is used to solve longstanding trait-function puzzles, such as the decline of photosynthetic nitrogen use efficiency with leaf age and the high variability in nitrogen resorption at the end of leaf life.**

Photosynthetic potential declines through leaf life, before senescence begins, and without commensurate decline in the N concentration (*8, 10, 33*) (Figure 1). Resorption of N during leaf senescence is fairly inefficient on average (~50%) (*34, 35*) and varies widely (14-83%)(*36*). Also, N resorption does not appear to be greater where N should be more valuable to the plant, for example on low-nutrient soils (*11, 34*). Leaf N reallocation to new growth is less than canopy-optimization models predict (*33, 37-40*). Species with long leaf lifespans and high leaf mass per area tend to have lower photosynthetic nitrogen use efficiency (*2, 41-43*).

This proposal suggests that N resorption may be limited by the high cost of hydrolysing refractory proteins, particularly stress-related proteins, including defense-related proteins, some of which have evolved with the very purpose of resisting enzymatic degradation by proteases. As leaves in the wild come under pressure from microbes and herbivores, leaf N is allocated to stress-related proteins at the expense of photosynthesis-related proteins. Our hypothesis is that this process may account for the decline in photosynthetic nitrogen use efficiency as leaves age. Similarly, the wide range of leaf N recovered during senescence and recycled into new canopy leaves may reflect differences in the proportion of N in refractory stress-related proteins. Generally it seems likely that the defense and maintenance costs leaves bear, and their ecological consequences, may have been underestimated. Our understanding of leaf N up to now has been based mostly on observations of young healthy leaves, and technology has not previously allowed N allocation between photosynthesis and stress response to be quantified on an ecologically relevant scale.

Under Approach and Methodology below these hypotheses are translated into a sequence of specific questions. More broadly we suggest the use of proteomics to answer large-scale plant ecology questions will be a fast-developing trend.

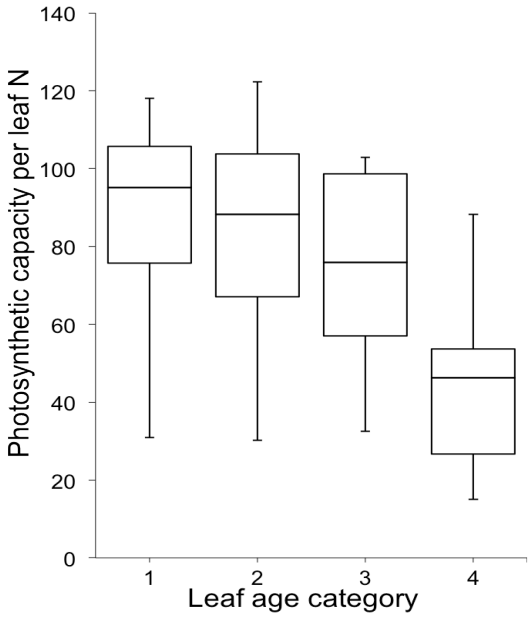

Figure 1. Leaf photosynthetic nitrogen use efficiency declines with leaf age. Average efficiency values (A_{max}/N (μ mol CO_2 s^{-1} mol N^{-1}) for 10 species at 4 leaf ages were derived from (10) (N data unpublished). Boxes: median, Q1, Q3; whiskers: min and max.

Stress- and Defense-related proteins

The distribution of species along the spectrum of growth rate, leaf longevity, and leaf mass per area (*1*) is influenced by pressures such as resource availability and defense requirements (*44, 45*). For example, in competition models that do not include biotic threats, plants evolve to have lower and lower leaf mass per area to maximize C assimilation per N (*46*), a scenario that contradicts the wide range of observed values. We will quantify expenditures on stress- versus photosynthesis-related proteins in order to better understand species traits, physiologies and physiognomies, and their interactions with the environment.

Protein level plant responses during plant-microbe interactions are highly complex (*47, 48*), particularly detection and signalling. Successful pathogens evade detection or suppress plant resistance responses. In natural vegetation leaves are constantly exposed to dozens of microbes at a time (*49, 50*), but they resist infection by the majority (*47*). Similarly, plants are regularly pressured by many different folivores, but most are resisted as shown by the abundance of leaves in most ecosystems (*18*). Considering that the protein machinery of photosynthesis is inherently nutritious, plants must allocate resources to defense so that the costs to other organisms of acquiring leaf nutrients exceed the benefits. Those defenses are both constitutive and inducible and include cell walls, lipids, toxic secondary metabolites, and inhibitory proteins.

We will assess how photosynthesis-related proteins and stress- and defense-related proteins change with leaf age to determine the cost of maintenance and defense in terms of protein nitrogen. To do so we will quantify the relative abundance of the protein categories using quantitative proteomics based on functional categories as described in "Approach and Methodology" below. Separately, structural proteins, will be assessed by measuring insoluble N because they represent a pool of refractory N that could change with leaf age (*51*), although some structural proteins are known to be hydrolysed during N resorption (*16*).

A subset of defense-related proteins is the pathogenesis-related proteins, comprised of 17 functionally and structurally distinct families, many of which inhibit microbial or insect growth (*52-54*); e.g. chitinases, thaumatin-like proteins, β -glucanases, lipid transfer proteins. Gene family sizes vary by species. For example *Arabidopsis*, rice, and poplar genomes contain 21, 30, and 51 thaumatin-like genes, respectively (*55*). Members of several families resist hydrolysis by proteases (*56-58*) and have been described as "hyperstable proteins" (*59*). This potentially benefits the plant through preserving defense proteins under the highly proteolytic conditions at plant-pathogen interfaces (*60-62*) and folivore guts (*59*). In fact, some pathogenesis-related proteins inhibit insect and pathogen proteases (*53, 63*). The proteolysis resistance of pathogenesis-related proteins has been linked to human food allergies because they survive the stomach (*64*). For the same reason, they accumulate in insect frass (*59*) and are believed to affect forage digestion in ruminants (*22*).

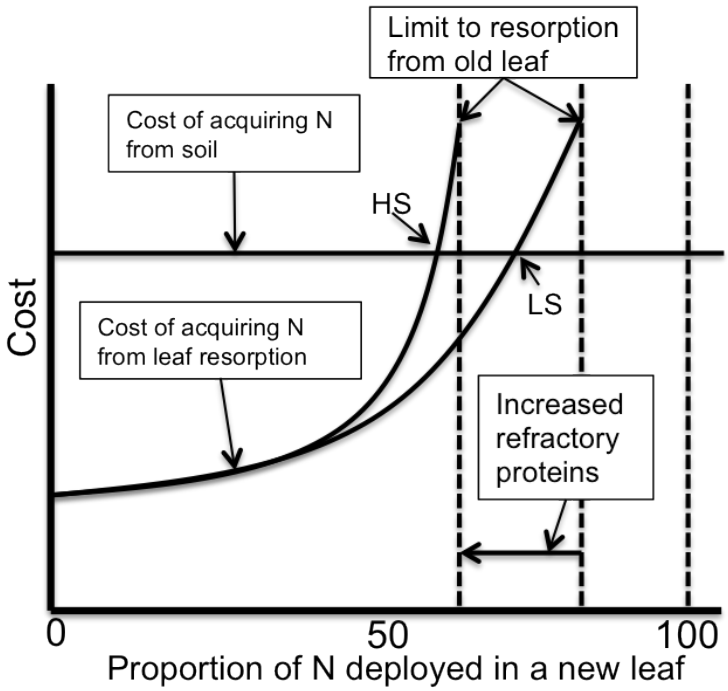

Figure 2. A model of N allocation to new leaves based on a fixed cost of N from soil and an increasing cost of resorption from aging leaves. As the resorption cost approaches the limit of recoverable N it exceeds the cost of soil N. The intersection is the projected resorption rate. Increases in refractory proteins reduce the limit of recoverable N. LS and HS (Low and High Stress) indicate projected resorption rates for leaves with low and high levels of refractory proteins, respectively. (Modified from (11)).

The downside of protease-resistance defense-related proteins is that their N cannot easily be reallocated to new growth. In addition to being induced by biotic stress, pathogenesis-related proteins accumulate in response to wounding and abiotic stress (osmotic, temperature, oxidative), and during senescence (*54, 55, 65-67*). As leaves age they inevitably are exposed to stimuli that induce expression of defense-related proteins. Abiotic stress has also been shown to reduce photosynthetic nitrogen use efficiency (*32*) and N resorption during senescence (*68*).

Stress

We use the term 'stress' for the sake of brevity—it pools a lot of phenomena on both the cause and effect sides. There are many complex mechanisms between the causes and effects of different stresses, but that is not what we are interested in. Instead, we are interested in the cumulative results of all the stresses, both abiotic and biotic, leaves face as they age.

Many abiotic stresses result in oxidative stress at the cellular level. Also, coping with oxidative stress is a universal necessity for leaves because reactive oxygen species are produced during photosynthesis and respiration (*69*). Abiotic stress, such as temperature and drought, increases

levels of oxidative species. In response, proteins involved in protection from oxidative stress and concurrent repair are up-regulated (*70*). We hypothesize that, as leaves age, N is increasingly allocated to stress-response and repair proteins at the expense of photosynthesis-related proteins. It is likely that some of those proteins are not protease resistant and, therefore, are recycled during recovery from stress. However, it is possible that some stress-related proteins are resistant to proteases so that they can function in damaged cells in which organellar compartmentalization is partially compromised. Protease resistant stress-related proteins would contribute to the pool of refractory proteins in the same way as protease resistant defense-related proteins.

If stress-induced proteins are protease-resistant, then the high cost of recovering their N could exceed the cost of N from soil uptake, resulting in refractory proteins accumulating in aging leaves. When N is limiting, stress-related protein accumulation will occur at the expense of photosynthesis-related proteins, thus reducing photosynthetic nitrogen use efficiency. Stress- and defense-related proteins are therefore credible candidates for a substantial pool of nitrogen that is not able to be resorbed by plants as leaves age or reach the end of their lives. Our hypothesis is modelled in Figures 2 & 3.

C3. RESEARCH PROJECT APPROACH AND METHODOLOGY

This proposal asks 6 questions. We feel confident about answering Q1-Q3 within 3 years and optimistic about Q4-Q6. The first question is about leaf aging, ***Q1: As leaves age (but while still alive and photosynthetically active), does the amount of leaf N in refractory stress- and defense-related proteins increase, at the expense of photosynthetic proteins, and potentially account for the decline in photosynthetic capacity with age?*** Given the wide variation between species in the fraction of leaf N that is resorbed at the end of life and recycled into new canopy leaves, we ask also ***Q2: Do differences among species in fraction of leaf N resorbed reflect differences in the proportion of N that is in refractory proteins towards the end of leaf life?***

Measurements of leaf N, insoluble N, protein abundance by functional category, photosynthetic measures (A_{max}, dark respiration), and herbivory and microbial damage will be spread across phylogeny and latitude so that broad trends may be observed and globally relevant conclusions reached. Evergreen C_3 species that do not fix N will be used for leaf sampling from at least 10 species per site using 8 established Westoby sites ranging from Tasmania to Far North Queensland (e.g. (*71*)). Photosynthetically active leaves of 3-4 age categories and senescent leaves will be collected and ages determined as in (*72*). For 15 species per site, 3 leaf ages plus senescent leaves, and 3 replicates, there would be 1440 samples; 10 species per site would be 960, and the number could be as high as 1800 with increased numbers of leaf ages, replicates, or species.

A wide sample range is necessary because the goal is not to elucidate detailed biochemical pathways of stress response as is often done with model species, but instead to improve ecological models of N usage by accounting for declining photosynthetic capacity with leaf age (Q1) and the broad range of reported leaf N resorption rates that to date cannot be explained (Q2).

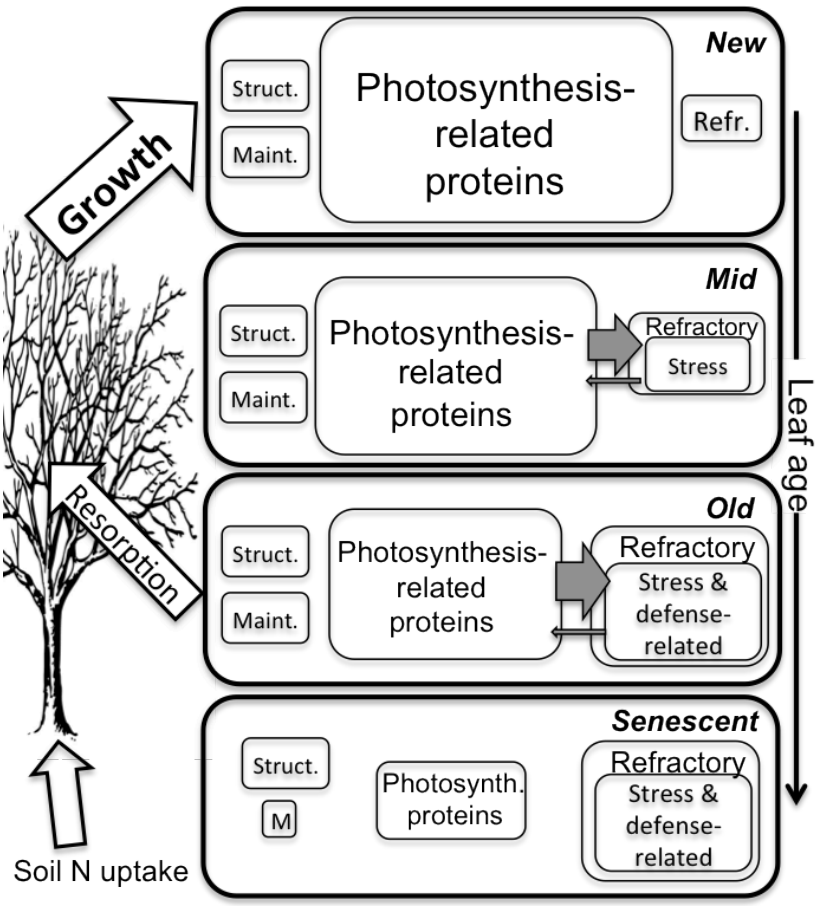

Figure 3. Hypothesized relationships among pools of protein nitrogen. Light arrows indicate acquisition of N and allocation to new growth. The four large boxes represent leaves aging from top to bottom. Dark arrows within leaves indicate reallocation of N during stress (large arrows) and recovery (small arrows). The majority of proteins in young leaves are photosynthesis-related, mostly rubisco. As leaves age and experience stress, N is allocated to stress- and defense-related proteins. Because some stress- and defense-related proteins are resistant to proteolysis, N is not fully returned to photosynthesis-related proteins during stress recovery. Thus, N accumulates those refractory proteins as leaves age. A high proportion of N in senesced leaves is likely stress- and defense-related proteins. Similarly, other refractory proteins, regardless of functional group (e.g. structural proteins), might accumulate with age, also.

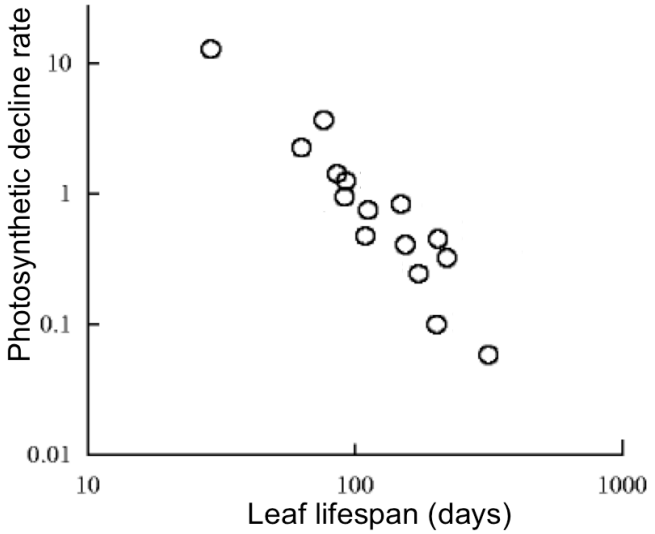

Figure 4. The decline of photosynthetic capacity with leaf age is faster in species with shorter leaf lifespans (rate: nmol $CO_2 g^{-1} s^{-1} day^{-1}$). Each point represents a different species (from (8)).

The relative decline in photosynthetic capacity is just as great in short-lived as in long-lived leaves, in other words photosynthetic capacity declines faster in species with short-lived leaves (Figure 4). Hence ***Q3: Do the N-pool changes associated with leaf aging (as hypothesized under Q1) come about faster in short-lived leaves?*** One possibility is that species with short leaf lifespans accumulate stress- and defense-related proteins faster. That might occur if stress responses were stronger in short-lived leaves, reallocating N away from photosynthetic proteins faster than for long leaf lifespans. Alternatively, species with short-lived leaves might resorb nitrogen from them faster for reallocation in new growth. In that case, the decline in total leaf N would be more in line with declining photosynthetic capacity (A_{max}) in species with shorter leaf lifespans.

Q4: Do differences among species in N resorption efficiency and proficiency reflect different fractions of N placed in constitutive defenses from the outset of the leaf's life? This question, related to Q2, will be answered by comparing relative abundance of defense-related protein in new leaves with N levels in senescent leaves (resorption proficiency) and with the percentage of total N in senescent leaves relative to total N in new leaves (resorption efficiency). Answering this question accounts for the possibility that differences in N resorption are not a reflection of accumulated, induced defense-related proteins, but are instead simply related to what proteins the leaf had to begin with.

Because defense-related proteins are induced by abiotic factors, we will avoid sites known to have experienced unusually severe weather during the maximum leaf lifespan prior to sampling. Old leaves commonly show sign of herbivory or plant response to microbes (necrotic or chlorotic lesions). We will visually score sampled leaves for herbivory, necrotic/chlorotic spots, and visible evidence of microbes (e.g. mildew) so that microbe/herbivore pressure can be related to defense-/photosynthesis-related protein ratios at 3 or more latitudes.

N:P ratios in leaves and leaf litter are negatively correlated with latitude (Figure 5, (*9, 14, 73*)), suggesting that species from lower latitudes have relatively higher levels of proteins and lower levels of RNA than species at higher latitudes. Because photosynthesis-related enzymes are more efficient at higher temperatures (*74*), it is counterintuitive that species in warmer climates have higher leaf N:P, although the trend could reflect soil N:P (*14*). Intriguingly, although photosynthetic nitrogen use efficiency has been observed to increase with decreasing latitude in young leaves (*75*), plant productivity per N decreases with increasing mean temperature at the community level (*73*), raising the possibility of more dramatic declines in efficiency with leaf age at lower latitudes. Potentially those declines could be caused by increased biotic stress in the tropics, consistent with a higher abundance of microbes at lower latitudes (*49, 50*).

Lower levels of RNA per N (RNA being the largest pool of mobile P (*76, 77*)) suggests that protein turnover is lower at lower latitudes, a potential indication that the higher N levels are related to accumulated protein rather than to a dynamic pool. The finding that leaf litter N:P is substantially higher at lower latitudes is consistent with efficient resorption of P and inefficient resorption of N relative to higher latitudes (*78*). Lower resorption efficiency of N, we hypothesize, is related to higher levels of refractory proteins, which is consistent with the observation, based on protease spiking experiments, that leaves from lower latitudes contain lower percentages of digestible protein than those from higher latitudes (*24*). Hence ***Q5: Are ratios of stress- and defense-related proteins to photosynthesis-related proteins higher at lower latitudes?***

Established sites of the Westoby group are spread across aridity as well as latitude gradients (*71*). By sampling at those sites we hope to be able to answer ***Q6: Do the ratios of stress- and defense-related proteins, and photosynthesis-related proteins differ with aridity?*** High leaf N concentration is associated with high aridity, a strategy believed to increase CO_2 drawdown by rubisco per stomatal conductance (*72*). On the other end of the aridity spectrum, it is possible that wetter conditions promote microbial pressure leading to higher levels of defense-related proteins.

The questions above are ranked roughly in the order of the likelihood they will be answered. We are confident Q1, Q2, and Q3 will be answered by the methods we propose. In Q4 our ability to determine constitutive defense-related protein expression is dependent upon the level of noise introduced by microbes and herbivores. Successful answers to Q5 and Q6 depend upon our ability to process enough samples across broad enough gradients to establish trends.

We have established sites in Australia and international collaborations that could provide enough suitable samples, but our ability to compare protein abundance is limited by our sample processing capabilities. We expect that 1000 total leaf samples should give us a good shot at answering all 6 questions and consider that to be the minimum number of samples for the project. If everything goes perfectly, then the maximum number of samples is 1800 and that would increase the chances of conclusively answering Q5 and Q6. Because an ecological proteomics project of this scale is unprecedented, it is difficult to make predictions about adequate sampling for predicting trends on a global scale. 1000 samples are sufficient for Q1-3, but might not be for Q5 and Q6. Questions 1, 2, and 3 are the most important questions in this project; they answer significant longstanding ecological questions and are independent of Q4-Q6.

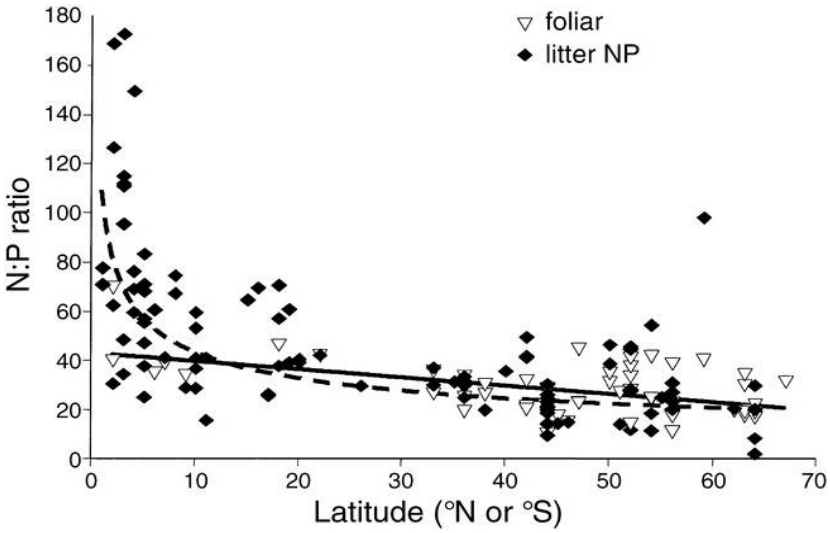

Figure 5. N:P increases with decreasing latitude. Differences in litter N:P are particularly striking and might result from abundant refractory proteins in senescent leaves. (From (9))

We emphasize that the proposed work is not a one-off project. Mass spectrometry is an area of excellence for Macquarie University that has not been exploited for plant ecology research. We expect to open up a productive line of research, made possible by quantitative proteomics, that we intend to pursue beyond Q1-6 and leaf aging.

PROTEOMICS

This proposal is possible because of the mass spectrometry resources available at Macquarie University in the Department of Chemistry and Biomolecular Sciences and the Australian Proteome Analysis Facility. Equally critical are the data analysis experience and capabilities of CI Haynes and PI Gygi. Proteomic analysis of 1000-1800 samples in 3 years is an imposing task, and is unprecedented in ecology, but current large-scale medical proteomics projects include tens of thousands of samples. One example is the current human interactome project of the Gygi group, which is analysing >30,000 samples to investigate the interaction partners of ~10,000 human proteins (*79*). For the proposed plant ecology project, like other proteomics projects of large scale, the major challenges are data analysis and sample preparation—mass spectrometry instrumentation is not limiting and the fieldwork is straightforward.

Our solution for analysing the large amounts of data generated by this project is to utilize the computational resources of the Gygi group, which are already in place and are accessible worldwide through a web browser interface. The sample processing challenge is the main motivation for this proposal: to fund a full time Research Assistant to assist with sample collection and processing, and to cover associated consumables costs. In addition to the Research Assistant, we will also make use of an ARC LIEF-funded sample-processing robot (LE130100073) through our existing collaboration with Prof David Tissue (Univ Western Sydney).

Our connection with Tissue and the sample-processing robot is through a Science and Industry Endowment Fund (SIEF) John Stocker Postdoctoral Fellowship, awarded to Westoby, Haynes, Tissue, Dr. Belinda Medlyn (MU), and Dr. Ying-Ping Wang (CSIRO). The SIEF fellowship is funding CI Van Sluyter to perform leaf proteomics experiments with the aim of improving vegetation models based on N allocation strategies among protein functional groups. The SIEF project forms the foundation for the proposed project because it is establishing the proteomics methods for this project and it tests the underlying assumptions, such as defense-protein protease resistance, through more traditional biochemistry and physiology experiments. The proposed project builds from the physiological underpinnings of the SIEF project to expand our proteomics capabilities to large scale, longstanding ecological questions.

Mass spectrometry

The purpose of the mass spectrometry experiments are two-fold: 1) to quantify relative abundance of proteins by functional groups; 2) to quantify selected abundant proteins from each functional group in absolute terms (e.g. mg of a particular protein per leaf area). The two analytical aims require two analyses per sample: the first without internal peptide standards, the second with internal synthetic peptide standards for proteins of interest. In both cases proteins will be isotopically labelled with commercially available reagents called Tandem Mass Tags (TMT) from ThermoFisher. The TMT reagents fragment during mass spectrometry to produce reporter ions that are used to quantify differences in protein abundance among the samples. The current TMT regents allow simultaneous quantitative analysis of 8

samples per mass spec run. However, it is expected that new TMT reagents will become available in 2013 that will allow at least 10 samples to be run simultaneously, and so we expect to run approximately 10 samples at a time, including synthetic peptide standards (peptide standards are described below under *Absolute quantification using internal standards*).

The Gygi group recently discovered that established tandem mass spectrometry TMT methods (MS/MS or MS^2 methods) underestimate protein abundance ratios (*80*). For that reason we will use the MS^3 developed by the Gygi group to overcome the interference inherent to MS^2 methods, thereby measuring protein abundance ratios among samples with very high precision. The Australian Proteome Analysis Facility (APAF), hosted by the Macquarie University Department of Chemistry and Biomolecular Sciences, has two high-resolution instruments capable of the MS^3 method. One of the instruments, a Thermo Orbitrap Elite, is identical to those used by the Gygi group for TMT MS^3, and that is the instrument we will use on a fee basis. Other mass spectrometers within the Haynes group and the Department of Chemistry and Biomolecular Sciences will be used for preliminary analysis, mainly for assuring sample quality before using the Orbitrap Elite.

Quantification of proteins by functional groups

Protein functional groups will be compared at first, as opposed to individual proteins, because the purpose is to test ecologically based leaf N hypotheses rather than to elucidate the functions of individual proteins. Comparing functional groups of proteins will greatly simplify data analysis. The Haynes group, in collaboration with the Australian Proteome Analysis Facility, recently published one of the computational tools we will use, PloGO (Plotting Gene Ontology), that uses Gene Ontology (GO) annotations to group proteins into functional categories, then calculates changes in the abundance of groups (*81*); an example of its implementation is (*82*). A similar and widely used tool, BiNGO (*83*), has been used recently by the Haynes group (manuscript under review) and will also be used for functional group quantitation.

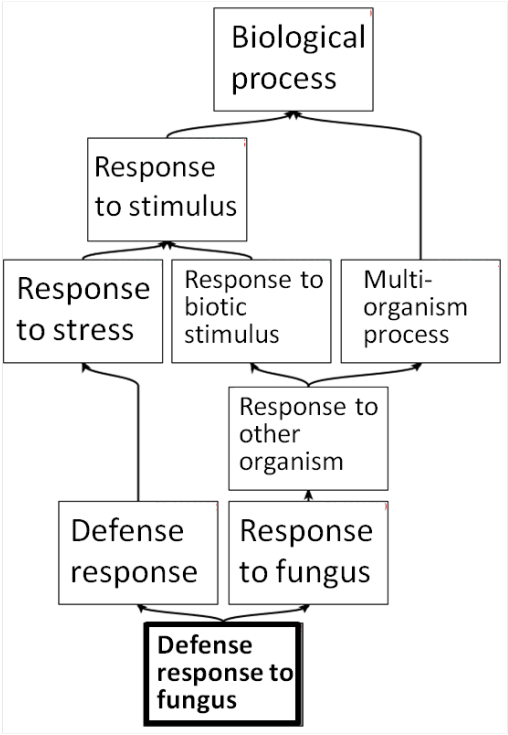

Figure 6. Chart of gene ontology (GO) annotations related to GO category 'Defense response to fungus.' Hierarchical relationships of GO annotations are used in PloGO to group functional categories. If 'Response to stress' were selected for quantification, then spectra matched to protein database entries assigned to that category, along with GO categories 'Defense response,' 'Defense response to fungus,' and other categories subordinate to 'Response to stress,' but not shown here (e.g. 'Response to oxidative stress'), would be summed for quantification as a group.

The widely used GO annotations are assigned with homology-based methods developed by a large consortium of functional genetics groups; most of the annotations were made computationally, linking protein sequences by homology to genes or proteins with experimentally determined functions (*84*). Tens of millions of plant protein GO annotations are publicly available (e.g. UniProt-GOA), and additional GO annotations can be easily created through freely available tools. Because GO annotations are related semi-hierarchically, it is possible to group them easily by hierarchy level (Figure 6). For the purposes of the proposed work, GO annotations will be grouped at very high levels, the result being very few categories for quantification: for example, defense-related proteins, photosynthesis-related proteins, response to oxidative stress, etc. Because of the potential importance of pathogenesis-related proteins, we will manually assign annotations of those 17 protein families.

Matching mass spectrometry data to proteins and functional groups is not possible without putative protein or gene sequences from the subject organism or a near relative. This project is only now becoming possible because of the recent rapid growth of available plant genome data. Most, if not all, of the species in our project will not have been sequenced. However, it will be possible to create the necessary protein databases using sequencing data from the same families as our target species. For example, *Eucalyptus grandis* genome data will be used for Myrtaceae samples. For Proteaceae we have arranged to use *Telopea speciosissima* from transcriptomics experiments conducted by Dr Paul Rymer (Univ Western Sydney) and Dr Maurizio Rossetto (Royal Botanic Gardens & Domain Trust, Sydney). Databases will be updated and refined as the project proceeds in order to keep up with the rapid growth of available plant gene sequence data. Haynes is experienced in matching mass spectrometry data from non-model species to nearby relatives for quantitative proteomics, for example using an *Arabidopsis* database for *Pachycladon* (Brassicaceae) data (*85*). Other examples include proteomics experiments with unsequenced sea urchins (*86*) and oysters (e.g. (*87*)).

Absolute quantification using internal standards

Using isotope labelling, MS^3 mass spectrometry, and functional categorization will allow precise measurement of changes in abundance among key classes of proteins in leaves. However, without the use of internal standards, the measured protein amounts are only relative among the samples, as opposed to being absolute. The potential pitfall of relative abundance measurements for trying to answer Q1-Q6 is that large apparent changes in relative abundance do not necessarily reflect large shifts in overall protein amounts. For example, a 10-fold increase in pathogenesis-related proteins with leaf age would potentially divert N from photosynthesis-related proteins if the pathogenesis-related proteins represented a significant portion of total leaf N (e.g. a shift from 1% to 10% of leaf N). Conversely, if the increase were from 0.001% to 0.01% of total leaf N, then that would not likely impact photosynthesis.

It is not possible to determine absolute protein amounts without internal standards. Therefore, for selected abundant proteins within important functional groups, synthetic peptide standards will be commercially synthesized, labelled with TMT reagent, and added to samples for a second MS^3 analysis (similar to (*88*)). By that method it will be possible to determine if the shifts in function groups determined in the first-pass experiment involve high enough amounts of proteins to affect photosynthetic nitrogen use efficiency and leaf N resorption efficiency.

Data management and additional statistical analysis

The experiments above will generate enormous amounts of data that will make additional hypotheses possible. To facilitate additional analysis all data, including leaf trait measurements, will be managed in a MySQL database with the assistance of Dr. Dana Pascovici (APAF). The database will make possible reorganization of data by Boolean searches for additional statistical analyses, such as multivariate analysis and cluster analysis at lower levels of the GO annotation hierarchy and protein levels. Also, protein sequences matched to experimental spectra will be grouped into clades by multiple sequence alignment for use in multivariate analysis based on protein structure. Protein localization and other homology-based predictions will be compared among the treatments to identify potential patterns.

Preliminary results

In a preliminary experiment, we compared levels of pathogenesis-related proteins in young, old, and senescent leaves from native vegetation in NSW. Leaves were collected from three wild species: *Lambertia formosa, Grevillea buxifolia*, and *Corymbia gummifera*. All three species at the site were found previously to decline in photosynthetic capacity with leaf age (*10*). Extracted proteins were subjected to short, 220 min low resolution LC-MS/MS analysis per sample without internal peptide standards. Proteins were identified using a publicly available database of 266,000 dicot proteins. Mass spectra were matched to between 1400 to 2000 proteins sequences per sample in the non-optimized database (protein false discovery rate of 2%, with a minimum requirement of 2 peptides matched to spectra per protein).

Proteins from 12 of the 17 pathogenesis-related families were identified and quantified in relative abundance using label-free methods. Chitinases and Thaumatin-like proteins were significantly higher ($p<0.1$) in older versus younger leaves in 2 of the 3 species. Pathogenesis-related proteins (~5 families in each of the 3 species) were proportionally higher in senescent leaves, as compared to old or young leaves, but quantification was not possible because internal synthetic peptide standards were not used. Similarly, proteins involved in oxidative stress response appeared to be higher in old versus young leaves, but they were not quantified.

The preliminary results demonstrate that the proposed methods can answer our six questions for three reasons:

1) Our sample preparation method is effective for challenging plant tissues (i.e. senescent sclerophyll leaves)
2) Mass spectrometry data for unsequenced native Australian species can be matched effectively to publicly available sequence data
3) Defense-related proteins do appear to increase with leaf age and senescence

We have found no evidence in our preliminary results or an extensive literature search that challenges our hypotheses. Our six questions are likely to be answered by the methods we have proposed, which are dramatic improvements over the limited sampling and technology used in the preliminary experiment.

SIGNIFICANCE AND INNOVATION

Unified theories of plant function supported by global trait surveys have emerged in the last two decades, but some traits remain for which convincing models do not yet exist (*45, 89*). Two trait-function relationships are addressed in this proposal: 1) the decline of photosynthetic capacity with leaf age beyond what can be explained by decreasing leaf nitrogen; 2) the variation in leaf nitrogen resorption from senescent leaves across species and environmental conditions. By addressing these two issues across a spread of species and habitats we hope also to explain interspecific differences in photosynthetic nitrogen use efficiency that occur in spite of highly conserved photosynthetic mechanisms.

Photosynthetic capacity is driven by abundant enzymes, particularly rubisco, which are limited not only by the costs of N acquisition from soil and existing tissues, but also by investments required for defense. Those defense costs can be thought of in terms of reallocating N away from photosynthetic capacity, but they have not been quantified on ecologically relevant scales. Abiotic as well as biotic stressors (e.g. drought, flood, increased ozone, temperature extremes) can potentially divert N from photosynthetic capacity to defense proteins. N allocation is expected to be influenced by climate change through several pathways.

Decline in photosynthetic nitrogen use efficiency with leaf age is a probable determinant of leaf lifespan (*33*). N allocation in general is linked to strategies of fast growth and short lived leaves versus defensive slow growth and long leaf lifespans (*44*).

Global biogeography is understood through models based on vegetation traits and C:N:P maps (*90*). The logical next step to improving models is to parse C:N:P, and that is what we will do with N. The ultimate goal of the work is to inform models, at all levels from the individual plant to the forest to the globe. Models of plant pests and climate change have focused mostly on the effects of climate change on crop pests, a common prediction being the spread of pests from lower to higher latitudes (*91*). Few models take into account the effects of plant pests on climate change. Predictions range from relatively benign to the dire case of a positive feedback loop between climate change and increased pathogenesis (*92*). The proposed project will elucidate the relationship between biotic stress and primary productivity.

The extensive use of protein mass spectrometry data in this proposal will represent a significant innovation for ecology. Ecological proteomics is only now becoming possible because of advances in data analysis methods and the rapid growth of genome sequencing data. A recent review (*93*) reported only 11 papers that used proteomics for plant ecology or evolution, each studying one genus at most. The present proposal will compare >60 species spread widely across phylogeny and geography. For proteomics, this proposal offers clear hypotheses that can focus the enormous amounts of data produced. Hypotheses about the ecology of nitrogen and proteomic technologies complement each other well. We expect the proposed studies to be the first of many.

NATIONAL BENEFIT

The project will answer fundamental questions with large consequences for how ecosystems work. This is relevant to National Research Priority 'An Environmentally Sustainable Australia: Responding to climate change and variability'. If we understood better the relationships among N, leaf lifespan, and photosynthetic capacity, that would be helpful for many applied ecology questions including impacts of elevated CO_2.

COMMUNICATION OF RESULTS

Results will be communicated through front-rank journals, plus conference presentations and invited seminars. Proteomics data will be uploaded to a repository such as PRIDE (www.ebi.ac.uk/pride). We plan to make the MySQL database publicly available subsequent to article publications.

C4. RESEARCH ENVIRONMENT

In the 2010 and 2012 ERAs Macquarie University achieved the highest ratings (4s and 5s) across many disciplines within Biological Sciences, including Ecology, Ecological Applications, Evolutionary Biology and Plant Biology. Ecology and evolution at MU includes strengths in plant ecological strategies, in biodiversity and conservation and climate change response, in conservation genetics, in microbial genomics and in evolutionary animal behaviour. A significant theme is international networking in order to build world-scale generalizations. This contributes to a high average citation impact per paper: in ecology and environment MU ranks 11th in the world and 1st in Australia among universities with >200 publications (Thompson-ESI 2001-2011). Seven Macquarie individuals rank in the top 400 worldwide for citations in ecology and environment. Labs besides Westoby's with interests related to this project include Prentice (global vegetation models), Medlyn (forest models and C-N relationships), Leishman (ecology of invasives), Wright (growth, respiration and trait ecology) and Hughes (plant-insect interactions).

In the proteomics area Haynes is a founding member of the Biomolecular Frontiers Research Centre (BMFRC), a university funded centre that brings together researchers with common interests in applying cutting edge technologies to the molecular analysis of biological systems. The mission of the BMFRC is to utilize systems biology, including genomics, proteomics, glycomics, and metabolomics, to increase understanding of physiology as a result of underlying genetic diversity.

Haynes has extensive experience in environmental proteomics, evidenced by his publication record and recent funding through ARC Linkage Projects and Discovery Projects related to environmental pollutants and climate change. The Haynes laboratory has two full-time dedicated high-speed ion trap mass spectrometry systems, funded by ARC LIEF grants, and there is an additional ARC LIEF funded high-resolution mass spectrometry system available within the BMFRC.

Co-located with the BMFRC is the Australian Proteome Analysis Facility (APAF), supported by ~$20 million (2007-2010) from the Australian Government through the National Collaborative Research

Infrastructure Strategy (NCRIS). The Haynes group works closely with APAF bioinformaticians (e.g. PloGO and other computational programs were written by APAF bioinformaticians to support Haynes's research). Mass spectrometry equipment and expertise critical for the proposed project is made possible technically and financially through past ARC grants and MU investments into BMFRC.

The MU Genes to Geoscience Research Centre is a federation across ~40 Sydney-region labs, including both Westoby and Haynes labs. Genes to Geoscience looks towards an emerging 4-way fusion among comparative genomics, functional ecology, earth system science and palaeobiology. To this end it organizes a yearly program of master classes for postgrads and postdocs. Through this framework CI Van Sluyter will communicate the new possibilities in ecological proteomics to wider audiences, and develop his teaching portfolio and mastery of plant ecology.

C5. ROLES OF PERSONNEL

This project will open up the new field of plant ecology proteomics, therefore it requires a new combination of skills and expertise. Our complementary combination is more than the sum of its parts because it utilizes experts in distinct fields to answer big questions in an entirely new way. Westoby is an experienced world leader in plant ecology. Van Sluyter is expert in refractory plant proteins, Haynes in the proteomics of plant abiotic stress, and Gygi in large-scale proteomics and bioinformatics.

Because of Westoby's depth of knowledge in plant ecology and expertise in leading ecology projects, he is the Project Leader. Westoby will supervise sampling design, project planning, data analysis and interpretation along with CI Van Sluyter. Haynes will be responsible for mass spectrometry experimental design and will oversee data acquisition and data analysis, which will be performed by CI Van Sluyter, the Research Assistant, and the PhD student. CI Van Sluyter and the Research Assistant will undertake all aspects of field and lab work. The Research Assistant is necessary for sample processing as described in the Budget Justifications. PI Gygi will provide access to his suite of proteomics data analysis tools and will contribute to experimental design and data analysis.

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